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CHROMOSOME ABERRATIONS AND LOSS OF SOME CELL FUNCTIONS FOLLOWING IN VITRO EXPOSURE TO RETORTED OIL SHALE

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ABSTRACT

An investigation of cellular level effects of processed oil shale from a simulation of modified in situ retorting was undertaken as part of an assessment of the toxicity and mutagenicity of oil shale. Complete assessment of the health hazards associated with physical contact, inhalation or ingestion of oil shale has not been examined in humans and until it becomes practical to assess these hazards in man, we must rely upon well established in vitro detection procedures in addition to whole animal testing. CHO cells and L-2 rat lung epithelial cell lines were exposed in vitro to processed oil shale particles at different intervals following exposure. Cells were analyzed for chromosome alterations, cell colony forming ability, DNA synthesis and cell transformation. The results of these studies demonstrate that retorted oil shale, under these experimental conditions, does modify cells in vitro. Chromosome aberrations increased with dose, cell colony forming ability decreased exponentially with dose, and the rate of DNA synthesis was affected, however cell transformation was not demonstrated after 3 months. Further studies are in progress. (This work was performed under the auspices of the U. S. Department of Energy.)

CHROMOSOME ABERRATIONS AND LOSS OF SOME CELL FUNCTIONS FOLLOWING IN VITRO EXPOSURE TO RETORTED OIL SHALE

Agnes N. Stroud

INTRODUCTION

There is concern over potential health hazards from pollutants formed as by-products in commercial production of energy. As the technology for processing new sources of energy becomes available, a variety of exogeneous agents will be introduced into the industrial environment which may be implicated in the etiology of occupational diseases, primarily lung ailments.

One potential source of energy currently under development is oil shale, which if commercially produced may raise industrial health questions. Some of the materials of concern are the polycyclic aromatic hydrocarbons and other organic compounds, organo-metallic compounds, trace metals, raw and processed shale, liquids and vapors, and other crude products.

Complete assessment of the health hazards associated with inhalation, ingestion or physical contact with spent oil shale has not been examined in humans and until it becomes practical to perform these studies in man, we must rely on well established detection procedures devised and refined by many researchers in a variety of *in vitro* and *in vivo* methods. Toxicological studies of oil shale can be assessed by *in vitro* methods in a way not feasible in an *in vivo* system. Therefore, we have undertaken a study to determine the effects of spent oil shale on cells growing *in vitro*. Three biological parameters which are important for survival, reproductive integrity (colony forming ability), chromosome stability, and DNA synthesis, were examined. These experiments will form a background against which the action of spent oil shale *in vivo* could perhaps be viewed with respect to lung tissue.

The results of these studies demonstrate that spent oil shale, in the form used and under the conditions of these experiments, does modify cells in an *in vivo* system.

METHODS

Cell Lines.

Two established cell lines which grow as monolayers in culture were used to evaluate the *in vitro* effects of the action of spent oil shale. The cell lines employed for different aspects of this study were CHO (Chinese hamster ovary fibroblasts) and L-2 (Fischer rat lung epithelial cells.) The CHO line was obtained from Puck[2] in 1962 and has been maintained and characterized at Los Alamos Scientific Laboratory by Deaven.[3] It has a near-diploid stemline of 21 chromosomes and for these experiments the cells were grown in Ham's F-10 medium (Microbiological Association, MBA), containing 50 units/ml Penicillin G potassium and 40 µg/ml Streptomycin sulfate. The L-2 cell line was obtained from Kaighn[4] at the 16th passage and at the time of these experiments had a modal chromosome number of 68. When the cell was cloned in culture it was characterized as a type II pneumonocyte.[5] This cell line was maintained on a medium modified by Kaighn[5] and designated F-12K medium (GIBCO), 15% fetal bovine serum (MBA), 50 units/ml Penicillin, and 50 µg/ml streptomycin.

Oil Shale.

The retorted oil shale was obtained from Laramie Energy Technology Center (LETC) after simulated *in situ* processing in a 150 ton retort. The shale was ball-milled to a dust ranging in size from 1.5 - 20µ, with the majority of the particles between 6 - 10µ. For use in culture, the shale was concentrated in a slurry in about 0.3 - 0.5 ml dimethylsulfoxide (DMBA) and diluted with 0.8% NaCl for sterilization by autoclave. Further dilutions were made in the appropriate media for adding to cultures. The oil spent shale in media was added to cultures immediately following the plating of cells. The pH of the media was not changed more than 0.4 on the pH scale.

Chromosome Analysis and Pulse Labeling with Tritiated Thymidine.

Cell suspensions, in their appropriate media, were inoculated into plastic T-25 flasks (Costar) at a concentration of 1×10^6 cells for chromosome analysis and pulse labeling studies. Oil shale (0.05 - 0.15 mg/ml) was added to three flasks for each dose following the inoculation of cells. The flasks were then incubated under 5% CO_2 and air at 38° C in a humid atmosphere. Chromosome

and DNA synthesis analyses were performed at 16, 22, 46, and 70 hours following treatment, and at these designated time periods, 0.1 µg/ml Colcemid (GIBCO) was added to each flask during the final three hours of incubation to block cells in division at metaphase. Twenty minutes prior to fixing cells, tritiated thymidine [^{3}H] TdR, in a concentration of 0.75 µCi/ml, was added to each flask to radioactively label DNA in the cells. Both chromosome analyses and labeling assessment were performed on cells which had been pooled from three flasks; however, separate slides were prepared for each. Chromosome spreads were made after the cells were treated with Colcemid, placed in warm hypotonic KCL (0.075) for 15 minutes at 38° C, and fixed in 3 changes of fresh cold fix, consisting of 1 part glacial acetic acid and 3 parts absolute methanol, at 4° C for about an hour. When the last fix was decanted, the cells were dropped from a micropipete on to chemically cleaned microslides which were removed from chilled distilled water (4° C.) The metaphase chromosomes spread on the wet slides after dropping, and to enhance the spreading, the slides were air dried by waving the slides in front of a hair dryer at 58° C for one half minute. They were stained with 4% Gurr Giemsa Stain (Improved R66) for 5 minutes. Between 100-200 V metaphase spreads (50 per slide) of good quality were analyzed for chromosome aberrations for each dose level.

Autoradiography was performed on cells which had been pulsed labeled with [^{3}H] TdR to determine the labeling index. For this analysis cells from fixative were dropped onto microslides and air dried. The slides were dipped in Kodak liquid emulsion (NTB) which had previously been diluted with equal parts of distilled water, and then stored in black slide boxes with a drying agent at 4° C for 7 - 10 days before developing with D19 developer and staining with 1% Gurr Giemsa. For each dose, and subsequent time interval, 500 cells were scored for incorporation of ^{3}H TdR into DNA.

Cell Survival.

Cell survival was studied by exposing single cells in culture to various doses of oil shale and scoring for visible cell colonies after a suitable period of incubation with the spent oil shale and subsequent removal of the agent. Spent oil shale (0.05 - 0.3 mg/ml) was added to plastic petri dishes (60 mm, Lux) which had previously been seeded with about 200 single cells. After an incubation period of 6 days with spent oil shale suspension the spent

oil shale was removed and the petri dishes were washed three times with Hank's balanced solution (GIBCO.) Fresh media was then added and the dishes were allowed to incubate 10 days at which time the cell colonies were fixed and stained with 1% Gentian Violet. Colonies containing 50 or more cells were scored and the mean number of 3 - 5 replicate dishes was determined. A survival curve was determined from the mean number of colonies formed from the single cells following treatment. Two or three experiments of the same type were performed at different times and the results were very similar, therefore, only one survival curve will be shown for the CHO or L-2 cell lines.

RESULTS

Cell Survival.

The response of cells to spent shale suspensions was studied by exposing single cells in culture to various concentrations of shale for 6 days, and scoring for visible cell colonies 1 week following the removal of spent shale. The survival curves for the cell lines CH) and L-2 are shown in Fig. 1. The colony surviving fraction for both cell lines show an exponential response resembling that of a single-hit kinetics[6] with an extrapolation number very close to 1. The mean lethal dose or the percentage necessary to reduce survival to 50% (LD_{50}) was 0.33 mg/ml for CHO cells and 0.14 mg/ml for L-2 cells indicating that the L-2 (rat lung epithelial cells) were more sensitive to the spent shale than CHO (Chinese hamster ovary fibroblast cells.)

DNA Synthesis.

CHO cells were pulsed labeled for 20 minutes with [^3H] TdR before the end of exposure to spent shale. The percent of cells incorporating [^3H] TdR is plotted as a function of the duration of oil shale in Fig. 2. The labeling index of the control cells was between 52% and 62% over a 44 hour period. The treated cells (all doses) showed a decrease in incorporation of [^3H] TdR at 17 hours and reached a plateau at 21 days; thereafter, no further decrease was seen. There was a significant difference between the controlled and treated cells; and between the lowest dose (0.5 mg/ml) and the two higher doses (1.0 - 1.5 mg/ml), but no difference between the two. It was interesting that the reduction of DNA synthesis to 40% at 24 hours for the treated cells did not go

below this fraction at 46 hours, indicating that there may be two cell types, one sensitive and the other insensitive to oil shale. The supression of DNA synthesis was significant but the degree of supression was not as great as one encountered with radiation and radiomemetic drugs. It was noted that the rate of DNA synthesis was somewhat reduced among the treated cells, as measured by grain counts, and the reduction was related to dose.

Chromosome Aberrations.

The scoring of chromosome aberrations were analyzed on CHO cells in metaphase after the cells were exposed to oil shale for 16, 22, 46, and 70 hrs. The frequency of aberrations is plotted against the duration of shale treatment in Fig. 3. The peak of aberration frequency for the two higher doses occurred at 16 hrs and was 13% at 0.15 mg/ml and 9.5% at 0.10 mg/ml. There was a 2 - 2.5 fold decrease in frequency at 22 hrs and thereafter, no significant change in the slope of the curves. At the lowest dose (0.05 mg/ml) the peak (6%) was not reached until 22 hrs after treatment and the curve remained the same at 46 hrs, and by 70 hrs the frequency had returned to control values. The accumulated data were combined for chromosome aberrations over the 70 hr exposure period and were plotted as a function of dose in Fig.4, and the accumulated data for the frequency of cells with aberrations over the same period were also plotted as a function of dose in Fig. 5. In both cases, the dose response curves were linear indicating that chromosome aberration frequency and cells with aberrations were dose dependent. Chromosome aberrations produced in CHO cells vary both in type and frequency. Some of the types observed are shown in Plate 1; (a) centric fusion, (b) chromatid deletion, (c) dicentric, and (d) badly damaged chromosomes. Not shown were isochromstid deletions, exchanges and translocations. There were an abnormal number of centric fusion types (a) where the centromeres appeared to be affected and two chromosomes fused at this junction. There was an exponential increase from 1% at control levels to 9.5% at the highest dose (0.15 mg/ml) of spent shale. Polyploid cells for all doses increased 2 - 2.5 fold over controls, but the increase was not exponential.

In one experiment with human cells, lymphocytes (leucocytes) from blood were grown in culture and exposed to spent shale (0.5 - 2.0 mg/ml) for 46 or 67 hrs before chromosome preparations were made. Metaphase cells were scored

for chromosome damage and the frequency of chromosome aberrations is plotted as a function of dose in Fig. 6. There is a linear dose response to spent shale between 1.0 and 2.0 mg/ml. There were very few chromosome aberrations 46 hrs following treatment.

Sister chromatid exchanges (SCE) were investigated in L-2 cells after spent shale treatment. The cells were exposed to shale at the beginning of the culture period and about 48 hrs later, chromosome preparations were made and SCE were scored. Table I represents the frequency of SCE following 0.10 mg/ml oil shale. The data show that spent shale was effective in increasing the production of SCE over controls and the SCE/chromosome was significantly higher in the treated compared to the controls.

DISCUSSION

It can be concluded that under the conditions of these experiments, the spent oil shale composite affected the reproductive integrity of the cells and the ability of cells to form colonies. L-2 cells were more sensitive than CHO cells. Chromosome aberrations were produced and DNA synthesis was to a certain extent impared in CHO cells. Mutagenicity was not shown after treating CHO and L-2 cells with different doses of oil shale and for different lengths of time.

It is not known what material in the reported oil shale is responsible for producing loss of some cellular functions *in vitro*, but it is possible that leaching out of a metal or metals from the composite, could be responsible.

The data in this report are preliminary and the effects of spent shale on cells in culture does not imply that spent oil shale may act similarily *in vivo* over an extended period of time even though chromosome aberrations were produced in lymphocytes of peripheral human blood *in vitro*. It should be noted that spent shales may have process specific characteristics in creating a biological effect and that the effects associated with any one type of spent shale cannot necessarily be considered as typical.

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FIGURE LEGENDS

Fig. 1. The survival curves for CHO and L-2 cells as measured by colony formation after exposure to oil shale. The mean lethal dose (LD_{50}) for the CHO line was 0.33 mg/ml and 0.14 mg/ml for the L-2 line.

Fig. 2. The percent of CHO cells labeled with tritiated thymidine [^{3}H] TdR as a function of the duration of oil shale following exposure to different concentrations of oil shale.

Fig. 3. The frequency of chromosome aberrations in CHO cells as a function of the duration of oil shale following exposure to different concentrations of oil shale.

Fig. 4. The accumulated frequency of chromosome aberrations in CHO cells over the 70 hr. exposure period to oil shale as a function of dose.

Fig. 5. The accumulated frequency of CHO cells with chromosome aberrations over the 70 hr. exposure period to oil shale as a function of dose.

Fig. 6. The frequency of chromosome aberrations in human leucocytes 67 hrs. after exposure to oil shale as a function of dose.

PLATE LEGEND

Plate 1. Types of chromosome aberrations produced in CHO cells following oil shale treatment. (a) centric fusion, (b) chromatid deletion, (c) dicentric, and (d) badly damaged chromosomes.

TABLE I

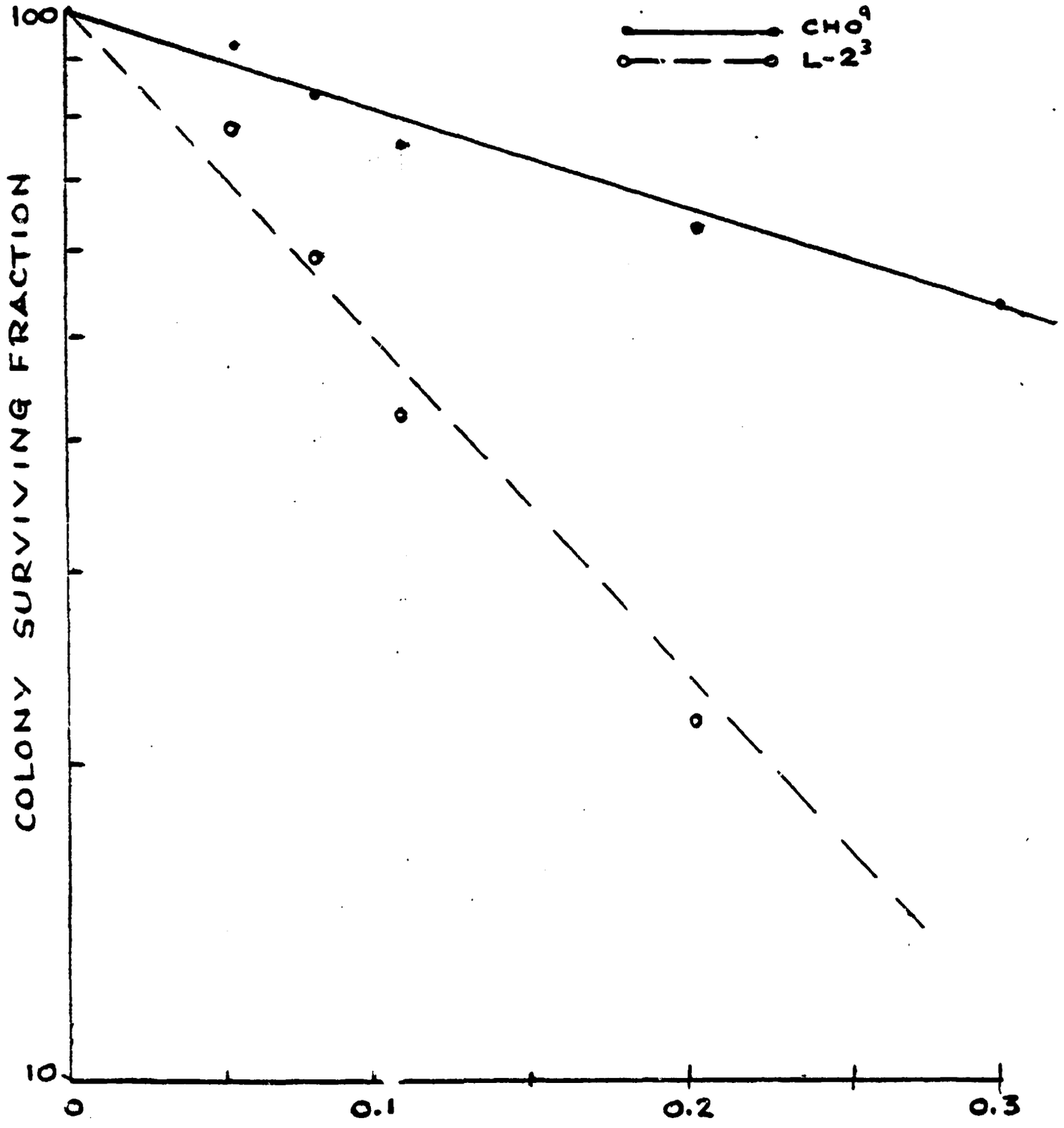
Sister Chromatid Exchanges (SCE) in L-2 Cells Following Exposure to Spent Oil Shale

Dose mg/ml	Chromosome Number (Mean)	Number Chromosomes Scored*	Total Number SCE	SCE Per Chromosome	SCE Per Metaphase (Mean)±
0	69	1718	209	0.12	8.4 ± 2.00
0.1	70	1813	311	0.17	12.0 ± 0.12

* 25 Metaphase spreads were analyzed at 0 dose and 26 at 0.10 mg/ml.

± Range of SCE/metaphase was from 5-13 for controls and 7-17 for the treated.

FIG 1



A. STROUD

FIG. 2

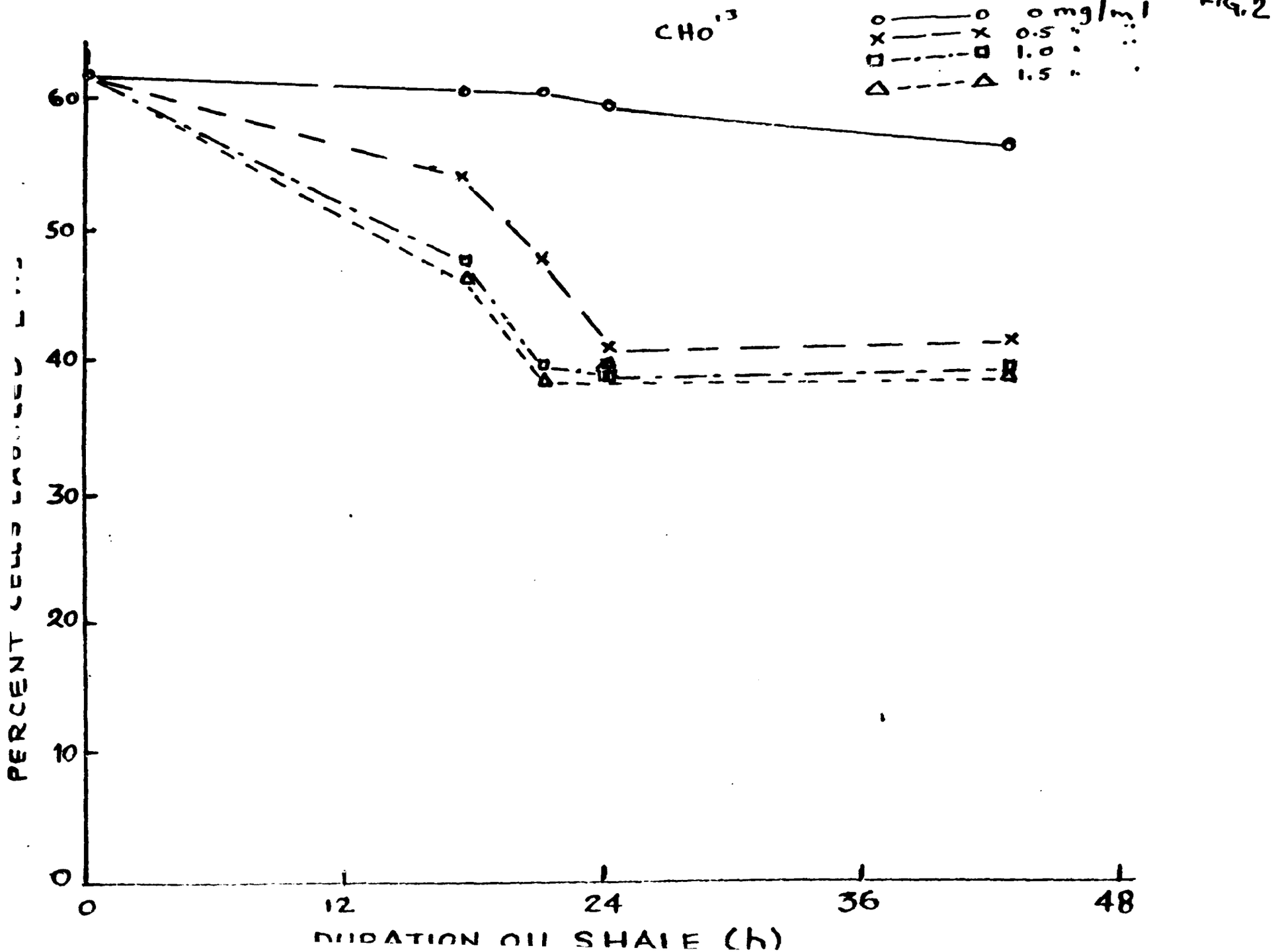
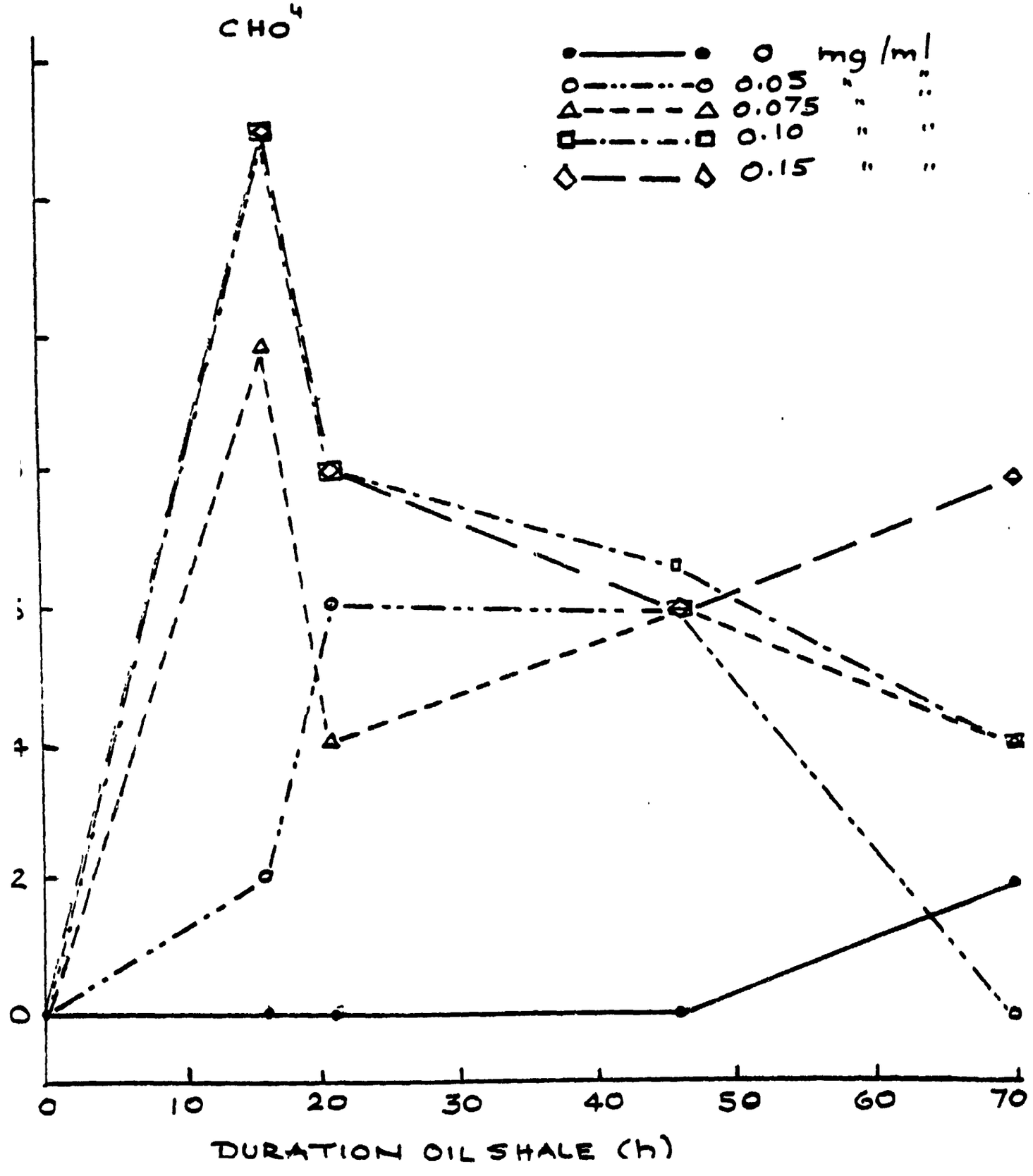


FIG. 3


CHO⁴
0 mg/ml
0.05 " "
0.075 " "
0.10 " "
0.15 " "
0
2
4
0
10
20
30
40
50
60
70
DURATION OIL SHALE (h)

A. STROUD
FIG. 4

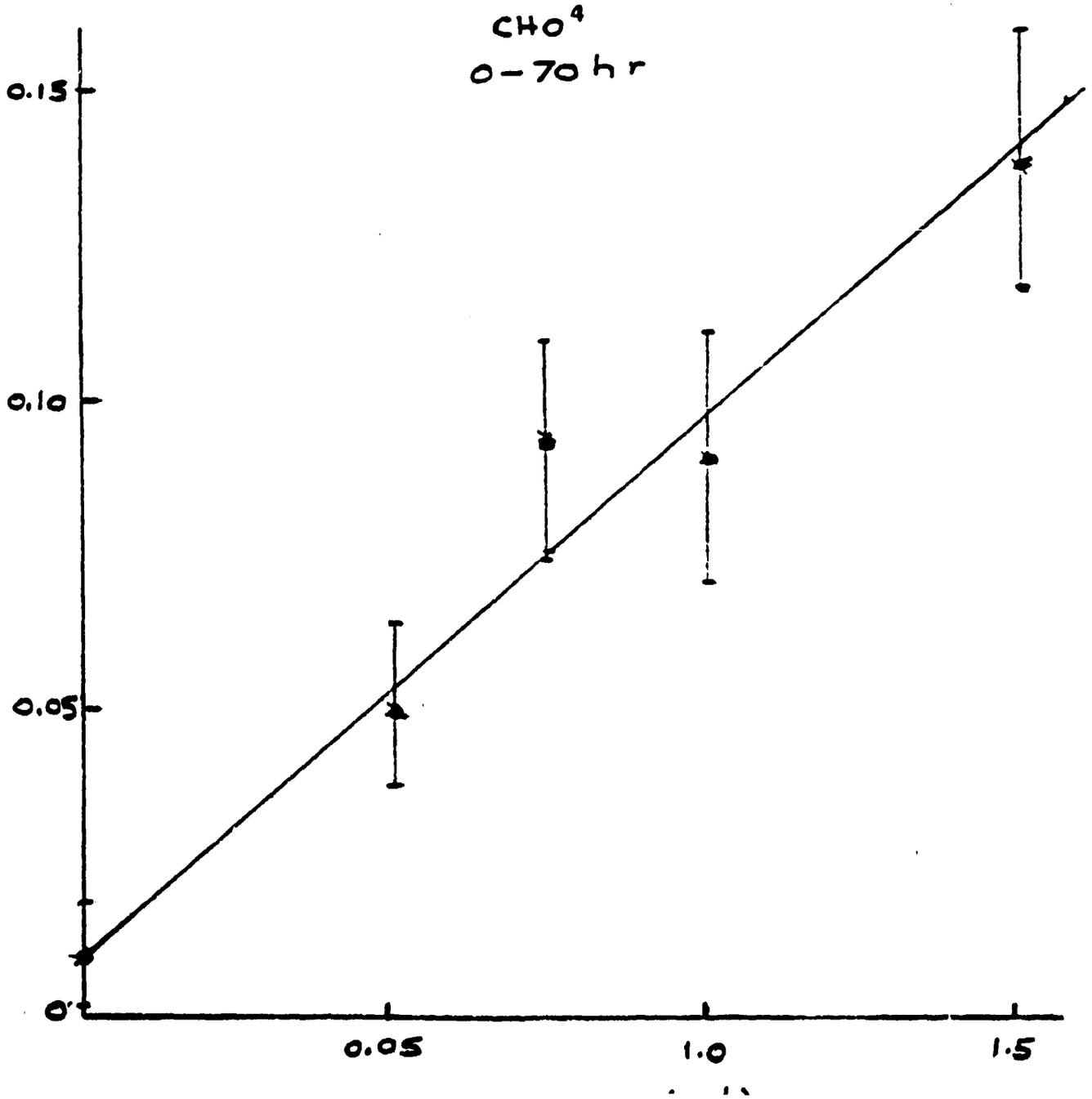
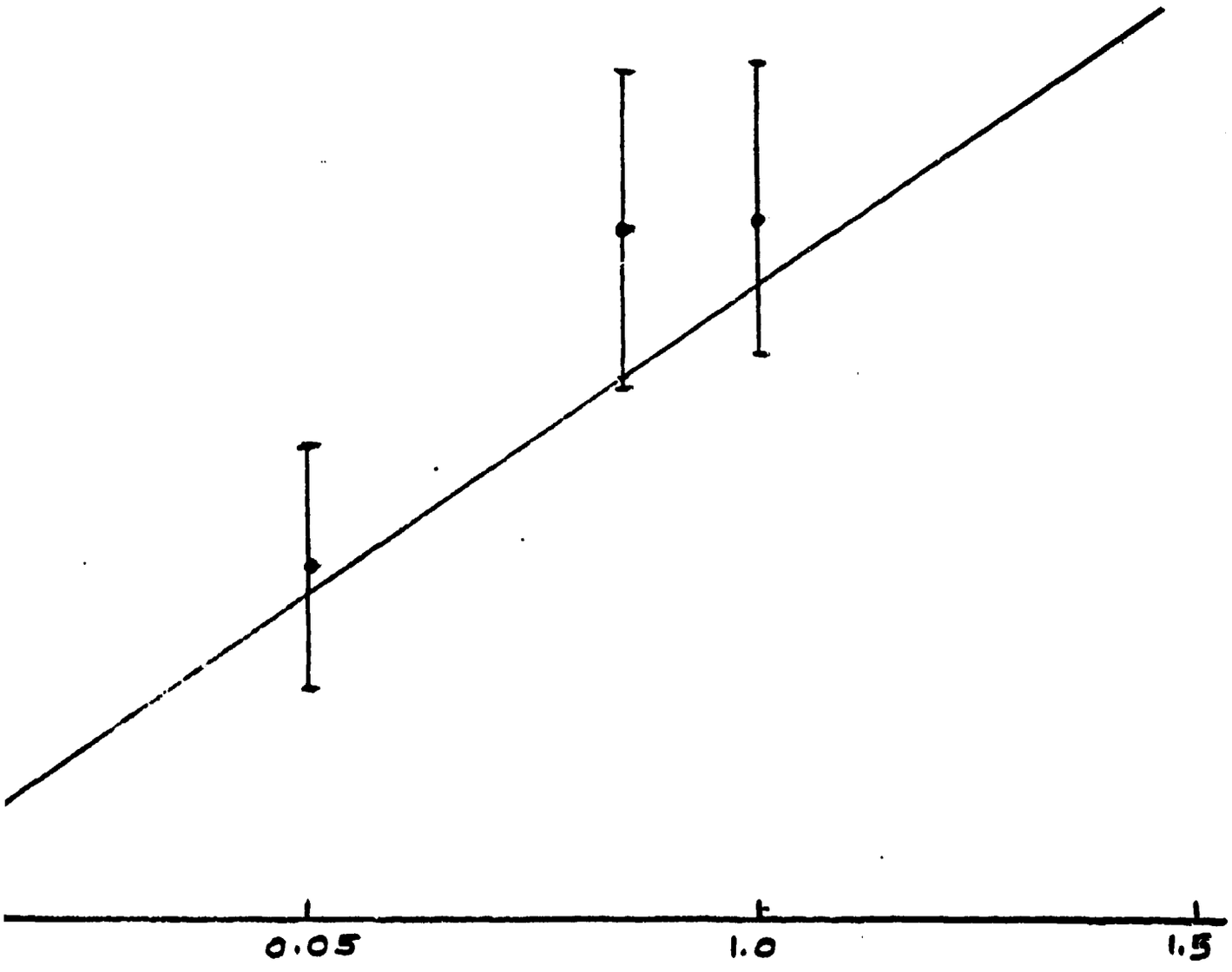


Fig 5

CHO[4]
0-70 hr



A. STROUD
FIG. C